



# Flow Cytometric MRD Assessment in Acute Lymphoblastic Leukemias

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Ind J Med Paediatr Oncol 2023;44:494–504.

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## Abstract

Acute lymphoblastic leukemia (ALL) is one of the very first malignancy where the assessment of early response to therapy by minimal/measurable residual disease (MRD) monitoring has proven to be cardinal tool for guiding therapeutic choices. At present, MRD detection is not only used for the assessment of initial treatment response and subsequent risk stratification but also for monitoring disease burden in the setting of hematopoietic stem cell transplant. Multicolor flow cytometry (FCM) for the assessment of MRD has been in existence for more than two decades. It is presently the most commonly used technique worldwide for MRD assessment in ALL. The technique has evolved from two to three color assays in its early phases to eight and more color assays in present time, which enables detection of one leukemic cell in  $10^4$  or more cells. The assessment of MRD is based on analysis of expression of lineage-associated markers and either looking at “leukemia associated immunophenotypes” or identify “different from normal” patterns. A rapid turn-around-time and direct quantification of viable residual leukemic cells are advantages of FCM over molecular techniques of MRD assessment. On the other hand, one of the prime limitations of detection of residual cells by FCM is the immunophenotypic shifts that are observed as a result of chemotherapeutic reagents. In addition, introduction of immunotherapy, especially against important gating markers like CD19, has posed significant challenge to FCM-based MRD assays, and requires modification of antibody panels for an alternate gating and analysis strategy. Finally, standardization and validation of MRD assay and use of internal and external quality controls are extremely important aspects for a clinical laboratory providing MRD reports for patient care.

## Keywords

- ▶ hematology
- ▶ acute lymphoblastic leukemia
- ▶ flow cytometry
- ▶ MRD

## Introduction

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm arising from the precursor lineage hematopoietic cells. The proliferation of these cells can be seen in bone marrow (BM), peripheral blood (PB), and extramedullary tissues. It represents the most common leukemia in childhood and can also be seen in substantial cases in adulthood.<sup>1</sup> Conventionally these are classified as either arising from B lineage (B-ALL) or

T lineage (T-ALL) precursor cells. Recently, there has been considerable research to subclassify these leukemias based on their molecular pathogenesis and this has become necessary in recent times due to their prognostic and therapeutic relevance.<sup>2</sup> In majority of the cases, the etiology remains unknown and ALL develops as a de novo neoplasm. The remaining includes either the genetically predisposing syndromes like Down syndrome, Fanconi anemia, few rarer entities like Bloom syndrome, ataxia telangiectasia, and

DOI <https://doi.org/10.1055/s-0043-1766137>.  
ISSN 0971-5851.

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Nijmegen breakdown syndrome or exposure to ionizing radiation, pesticides or solvents exposure, or infections like Epstein-Barr virus and human immunodeficiency virus.<sup>3</sup> Although chromosomal aberrations represent the hallmark of ALL, these, however, are not the sole pathogenetic etiology. Characteristic translocations especially in B-ALL include t(12;21) [*ETV6::RUNX1*], t(1;19) [*TCF3::PBX1*], t(9;22) [*BCR::ABL1*] and *KMT2A* gene rearrangements.<sup>4</sup> Accurate diagnosis of ALL by definition requires more than 20% blasts either in PB or BM<sup>4</sup> that is confirmed further for lineage assignment by multiparametric flow cytometry (MFC). Fluorescent in situ hybridization (FISH) and/or molecular tests like reverse-transcriptase polymerase chain reaction (RT-PCR) are required to subcategorize these cases for further prognostication and implications in therapy.

For the management of ALL, a prognostic evaluation is crucial. The clinical team can accurately determine the best course of treatment and the decision to consider the patient for an allogeneic stem cell transplant by an optimal risk stratification.<sup>5</sup> ALL is one of the first cancers where monitoring for minimal/measurable residual disease (MRD) has established itself as a crucial tool for determining the best course of treatment. Currently, MRD detection is used for initial treatment response evaluation, disease burden monitoring, and risk stratification in the context of hematopoietic stem cell transplant.<sup>6,7</sup>

## MRD in ALL

By definition, MRD refers to the presence of measurable tumor cells that are persistently present in a sample after the cancer treatment. While the assessment of morphological remission in ALL is still impeccable, the blasts are not always easy to distinguish especially when there is proliferation of hematogones, in the presence of regenerating normal myeloid precursors and after granulocyte colony stimulating factor therapy. Moreover, this morphological assessment is highly subjective and only a few cells are assessed at a given time.<sup>8-10</sup>

### Techniques for MRD

The most widely used techniques for analyzing MRD in ALL at the moment are MFC and PCR amplification based techniques, including the use of molecular markers specific to leukemia (fusion gene transcripts) or to the patient (rearrangements of the immunoglobulin/T cell receptor [TCR] gene). In addition, FISH cytogenetics, cell culture systems, Southern blotting, Sanger sequencing, and next-generation sequencing (NGS) are other modalities to assess the residual disease. PCR-based techniques have an advantage of high sensitivity that reaches  $1:10^4$ - $1:10^5$  (0.5-1.0 log higher than FCM). Furthermore, requirement of lesser amount of tissue compared with FCM is another important advantage of PCR techniques. However, this is a time-consuming methodology and requires highly specialized personnel for technical work, trouble shooting, and interpretation. Moreover, applicability of MRD analysis by real-time quantitative PCR (RQ-PCR) for fusion-genes is limited to only those cases that have a typical

translocation at diagnosis and is usually not helpful in ALL with a normal karyotype.<sup>11-15</sup>

### MRD by Flow Cytometry

MFC assesses the expression of differently expressed antigens by leukemic cells. Signals emitted by fluorochrome-conjugated monoclonal antibodies against these antigens are studied for MRD analysis. Two broad approaches have traditionally been in place to explain MRD assessment strategy, that is, leukemia-associated immunophenotype (LAIP) where the expression of antigens at diagnosis is tracked at subsequent time points on the residual leukemic cells; another method uses immunophenotypic shift in comparison to a normal counterpart population (hematopoietic progenitors of similar lineage and maturational stage) to identify leukemic blasts. This so-called "different from normal" analysis has the advantage of studying MRD without the requirement of knowledge of the diagnostic immunophenotype.<sup>16</sup> In real-world scenario, the boundaries between both these approaches are blurred, and a typical MRD-analysis is a combination of both of the concepts. FCM has the advantage of lesser turnaround time, its applicable in almost all ALL cases (up to 95%), and its readily available in routine hematology laboratories compared with other advanced molecular testing. In addition, a simultaneous assessment of cell qualities required for targeted therapies can be done. However, this technique also comes with its fair share of challenges. Immunophenotypic shifts are a known phenomenon postchemotherapy, which hinders the exact characterization of the leukemic cells on follow-up, most importantly when there is downregulation of the gating markers, like CD19 for B-lineage ALL. Steroids that are used extensively in all treatment protocols have been implicated in modulating the gene expression and hence alterations in immunophenotypic profile of the cells. Recent trends to utilize targeted therapy in ALL have also posed a challenge with respect to MRD assessment. Especially with B-ALL, there becomes a need to standardize a different antibody panel.<sup>17,18</sup>

## MRD in B-ALL

### Time Points for Assessment

For B-ALL, day 15 is considered as the first check point for the assessment of fast and early elimination of leukemic cells. Studies reported importance of MRD analysis at this nascent stage with patients having MRD negativity at this time point exhibiting an excellent prognosis.<sup>19,20</sup> The main advantage of day 15 MRD assessment is less interference from the proliferating hematogones as compared with the end of induction time point which needs a wider panel of antibodies to delineate these cells from leukemic B cells. Absence of hematogones increases the confidence of reporting with minimum set of markers and hence is more cost-effective specially in resource constraint laboratories.<sup>21,22</sup>

Nevertheless, most of the laboratories nowadays assess residual disease at postinduction, postconsolidation, and end of maintenance phases of treatment protocol. Each of

these time points exhibit prognostic relevance in risk stratification of patients. However, any suspicion of relapse or plan of change of therapy including decision for transplant warrants a more frequent assessment.<sup>23–26</sup>

### Technical Considerations

Heparin or ethylenediaminetetraacetic acid-anticoagulated PB or BM samples can be used. The BM sample has been shown to have more sensitivity to detect MRD cells in B lineage ALL, where the level tends to be 1 to 3 logs lower in PB than in BM. However, PB sample does have its advantage of being minimally invasive and hence more frequent monitoring is less cumbersome. Moreover, existence of PB-MRD positivity indicates high risk of relapse than isolated BM-MRD positivity. In fact, some studies have shown that a day 15 PB-MRD can provide an early, minimally invasive and easily accessible MRD screening option.<sup>27</sup> However, BM remains the sample of choice for B lineage ALL due to an overall higher detection rates than PB. This may not be true for T lineage ALL, where studies have shown a similar sensitivity for the detection of residual leukemic cells from PB and BM samples.<sup>23,28</sup> Minimal residual disease levels in BM and PB are comparable in children with T-ALL, but not in precursor-B-ALL.<sup>29</sup>

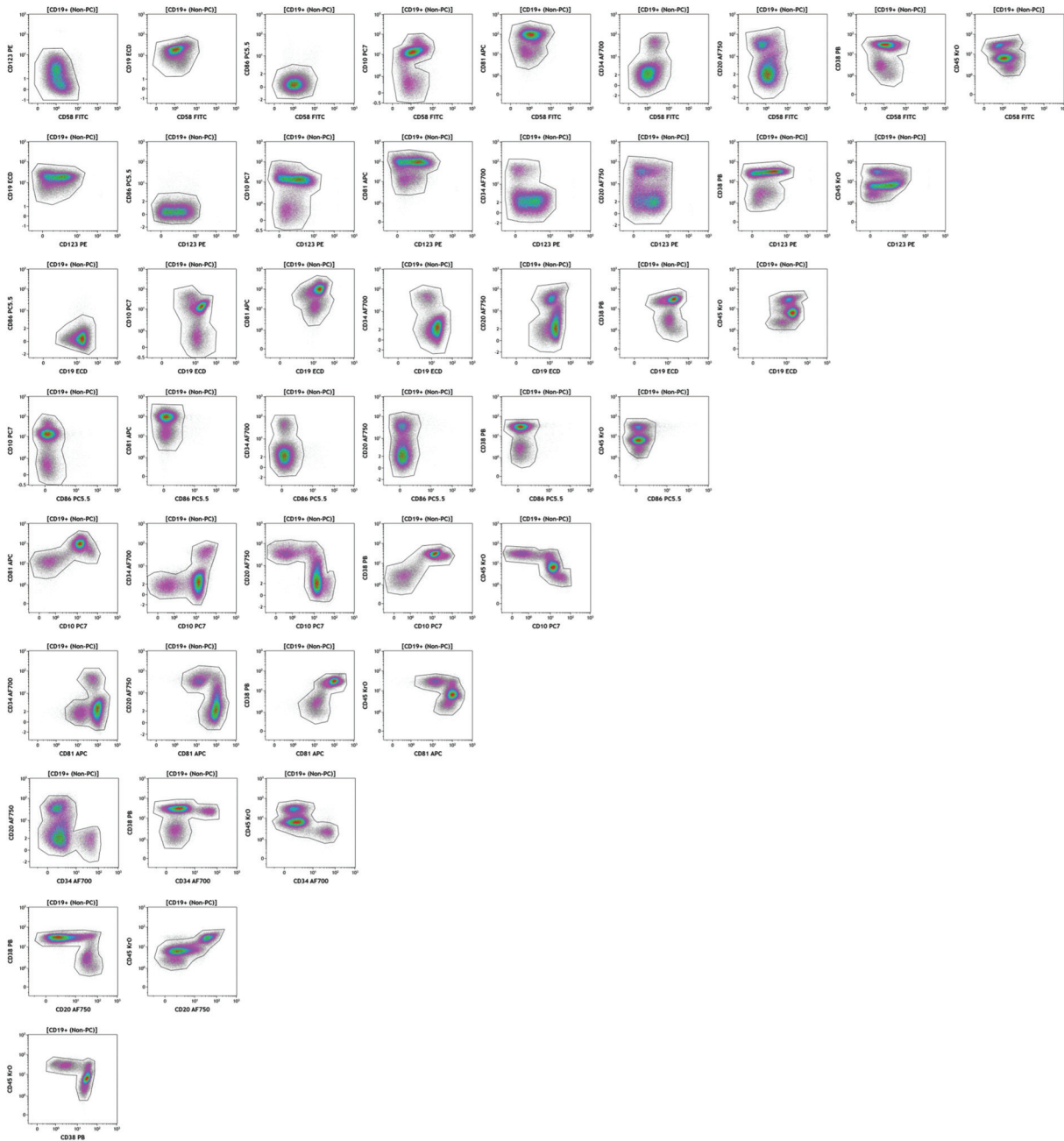
The optimal way to assess MRD is via an initial pull of not more than 3 mL of BM aspirate to avoid hemodilution of the specimen. Study by Helgestad et al showed 2.5 mL of first pull BM aspirate from one puncture site was appropriate for MRD analysis.<sup>30</sup> Samples should be processed within 24 to 48 hours and delivered ideally at room temperature. The EuroFlow group recommends “erythrocyte bulk lysis” procedure that seems to yield a higher number of leukocytes for acquisition of flow cytometer and hence likely to increase the sensitivity of the assay. Incubation with a prestandardized set of antibody cocktail(s) in dark, followed by thorough washing for removal of excess antibodies, should be done. The antibody cocktail should preferably of eight or higher colors for achieving a high sensitivity assay. A tube containing sheath fluid should be run prior to each sample run to avoid carry-over. To obtain a good sensitivity MRD data, acquisition of large numbers of cells becomes important. Acquisition of at least 1.5 million clean cells is recommended. With acquisition of more than or equal to  $4 \times 10^6$  the sensitivity of detection of MRD can be comparable to PCR-MRD data.<sup>31</sup> However, analysis of the data generated by acquisition of millions of cells, in a multicolor-high sensitivity assay settings, requires high-end computers with state-of-art processors, graphic cards, and advance data-analysis software. The high-sensitivity flow data analysis also requires special considerations. Careful removal of cell-aggregates, debris, and nonspecific events is necessary. Certain mimickers and artifacts can hinder MRD detection especially in high-sensitivity settings. Cells with overlapping immunophenotype like plasmacytoid dendritic cells, CD10+ transitional B cells, CD19+ natural killer (NK) cells, mesenchymal stromal/stem cells, as well as endothelial cells could well interfere with the rare true residual leukemic cells and should be kept in mind.<sup>32</sup> There is no clear recommendation

yet on the number of cells in a cluster required to define residual disease; however, most of the studies in B-ALL indicate a minimum number between 10 and 50 events.<sup>33</sup> Majority of the treatment protocols agree upon 0.01% clinically defined threshold to define MRD positivity. During the early phases of studies on clinical relevance of MRD in ALL, researchers from the International Berlin-Frankfurt-Munster (I-BFM) Study Group discovered that patients with MRD levels of 0.1% or above on both days 33 and 78 of therapy experienced a relapse rate of 75%, which led to the treatment of this particular patient population being intensified.<sup>34</sup> The Dana-Farber Cancer Institute ALL Consortium also noted an MRD threshold of 0.1% best predicted relapse hazard. The subsequent results with higher sensitivity assays documented the clinical relevance of 0.01% MRD cutoff, especially at end of induction time point.<sup>35</sup> The Children’s Oncology Group noted that the presence of MRD of 0.01% or greater, on day 29 was the strongest prognostic indication and predicted a worse outcome.<sup>20</sup> The UKALL2003 study group also showed benefits in event-free survival of augmented postremission therapy in children with presence 0.01% or greater MRD at the end of induction.<sup>36</sup> For the Indian Collaborative Childhood Leukaemia Group (ICiCle), developed in 2013, the cutoff for categorization into high risk group based on MRD levels at end of induction time point is 0.01%.<sup>37</sup> However, it may be relevant to note that depending on the protocol and on the time point at which MRD is examined, other threshold levels can also be informative.

### Strategy for MRD Analysis

FCM MRD in B-ALL was conceived in two or three-color flow cytometers that utilized patient-specific immunophenotyping to assess response compared with the baseline expression of the concerned markers. To enable pattern recognition in FCM plots and to detect aberrant regions showing MRD cellular phenotypes, various combinations of B-lineage maturation markers (CD10, CD20, CD22, CD19, CD34, CD38, CD45, and CD58) were utilized.<sup>38,39</sup> With further enhancements of the labeling capacities of cells, four- to six-color flow cytometers came into picture, thereby increasing MRD profiling capabilities and improving the sensitivity of MRD detection.<sup>40</sup> Recent efforts in standardization of MRD have shifted the capabilities of laboratories to an eight-color panel and beyond. For backbone markers, CD19, CD10, CD20, CD34, and CD45 are considered optimal. CD38 is also added and considered to be of high diagnostic relevance.<sup>41–43</sup> Other markers considered to be relevant for enhancing sensitivity of the assay include CD73, CD86, CD66c, CD123, CD81, CD44, CD58, CD9, and CD304.<sup>44–47</sup> CD15/NG2 might also be relevant in ALL with *KMT2A* gene rearrangements that is predominantly seen in infants. In addition, these cases are usually CD10 negative.<sup>48</sup>

The core approach of MRD analysis is to distinguish residual leukemic blasts from regenerative normal B cell precursors (BCP) in postchemotherapy setup.<sup>49</sup> The BCPs are known to follow a well-conserved immunophenotypic maturation sequence for the backbone markers viz CD10, CD19, CD20, CD34, CD38, and CD45. The premise for identifying

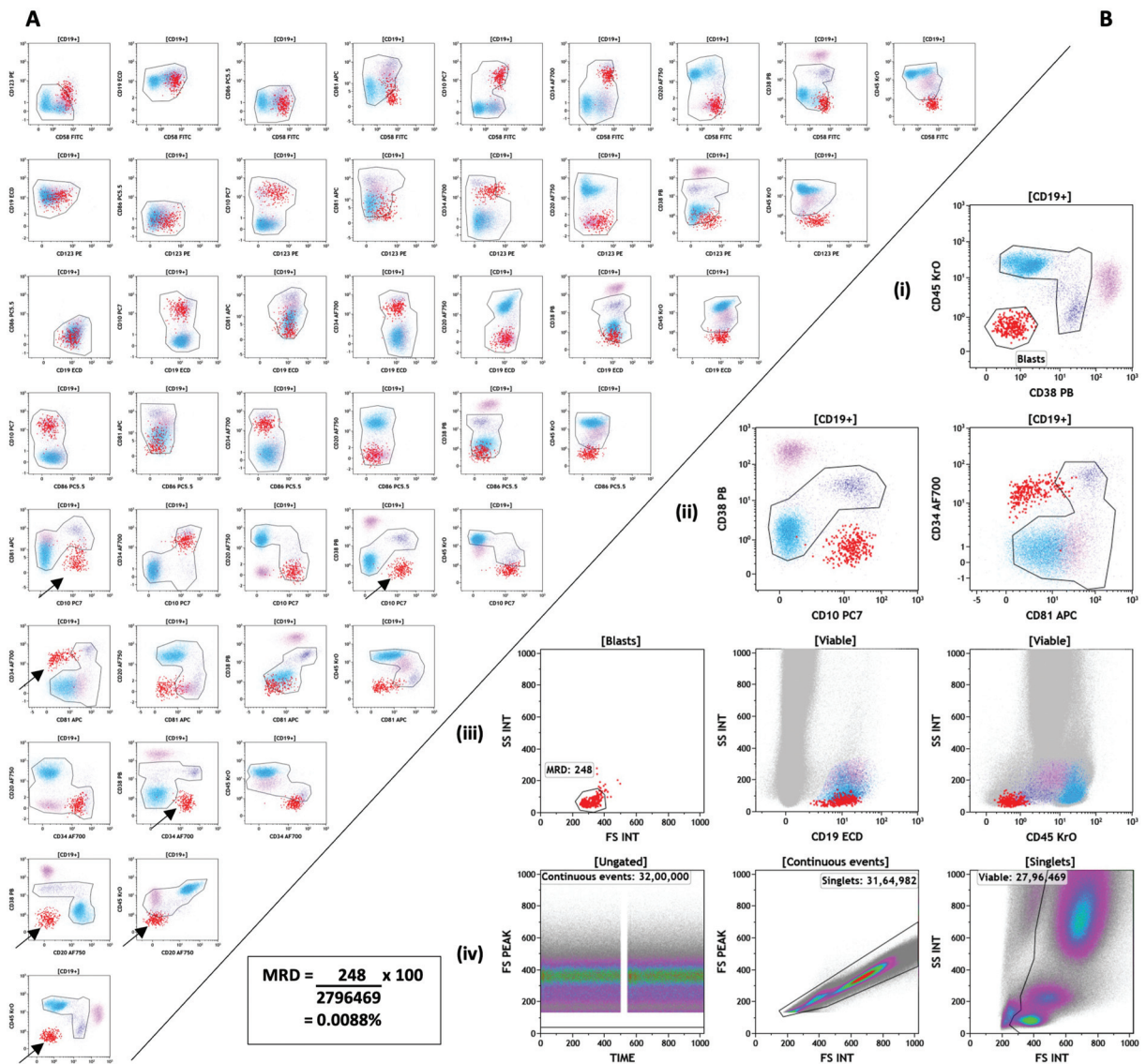


**Fig. 1** Panel of biaxial plots showing hematogone maturation pattern using a 10-color antibody cocktail comprising of CD58 FITC, CD123 PE, CD19 ECD, CD86 PCS.5, CD10 PC7, CD81 APC, CD34 APCA700, CD20 APCA750, CD38 PB, and CD45 KRo. The plots depict CD19 gated population of cells after removal of plasma cells to create a template for assessment of B-ALL MRD.

remaining leukemic cells is any variation from these normal expression or patterns brought on by overexpression, under-expression, or asynchronous expression.<sup>50</sup> Among these CD19 positive cells, plasma cells should be delineated based upon their CD38<sup>high</sup>, CD10<sup>neg</sup>, CD20<sup>neg</sup> expression. Hematogones proliferation usually occurs after consolidation therapy and is recognized by their specific patterns of maturation (► **Fig. 1**). Hematogones are usually CD81<sup>bright-pos</sup> and CD58<sup>moderate-pos</sup>. The early hematogones are CD123<sup>neg</sup> and CD86<sup>neg</sup>; however, late hematogones can show a dim expression of CD123 and CD86. Any deviation from these recognized patterns or maturation arrest hints toward a possible MRD (► **Fig. 2**).<sup>51</sup> Although CD34 is a marker for immaturity,

its expression is noted only in approximately 70% of cases of B-ALL.<sup>52-54</sup> Also, treatment may cause reduced levels of this marker, sometimes complete absence.<sup>55</sup> In addition, aberrant expression of myeloid markers, such as CD13, CD15, CD33, and CD66c, make these LAIPs useful for MRD evaluation.<sup>56,57</sup>

Newer parameters have been increasingly utilized to differentiate abnormal blasts from normal BCPs. Another effective marker for MRD diagnosis is CD73, which has great specificity and equal sensitivity to other common LAIPs. However, because of the bimodal nature of its expression in mature B cells, its interpretation must be done in the context of the expression of other markers. In hematogones,



**Fig. 2** (A) Panel of plots showing assessment of MRD on bone marrow aspirate sample from a patient of B-ALL at end of induction time point. The mature B cells are shown in light blue color, hematogones in dark blue, and plasma cells are in pink color. The events representing MRD are colored red and depicted in bolder dots than rest of the events. The MRD events are distinctly separate and lying outside the “hematogone-maturation pattern” in some of the plots (indicated by arrows), mostly in lower part of the panel. (B) Representative plots to show calculation of percentage of residual leukemic cells; (i) the residual blasts are gated on CD38 versus CD45 plot (red dots) where these show distinctly different expression for the two markers when compared with normal B cells; that is, CD10 versus CD38 and CD81 versus CD34, highlighting their deviation from normal maturation pattern; (ii) the gated residual blasts are shown in two other plots, that is, CD10 versus CD38 and CD81 versus CD34, highlighting their deviation from normal maturation pattern; (iii) forward scatter (FS) versus side scatter (SS) plot showing gating of tight cluster of cells to refine residual blast population which provides the numerator for calculating MRD, and a retrospective depiction of this MRD-cluster on CD19 versus SS and CD45 versus SS plots; (iv) the denominator for calculation of MRD is provided by gating viable cells on FS INT versus SS INT plot after gating continuous events on time versus FS PEAK plot and removing cell-aggregates on FS INT versus FS PEAK plot. The “text box” shows calculation of percentage of residual leukemic cells.

CD304 expression is weak and heterogenous. As B cells matures, its expression declines until it is completely missing. However, CD304 exhibits a robust expression when present on blasts.<sup>58</sup>

A common strategy is to create templates using the fixed scatter plots and predefined gates. These gates are designed after evaluating the normal BCPs on the treatment-naïve samples. End induction T-ALL, un-involved neuroblastoma/retinoblastoma, or non-Hodgkin lymphoma staging BM aspirate samples and BM samples from patients of immune thrombocytopenia are some of the scenarios where the

template for B-ALL can be made. Any “deviation- from-normal” is readily identified.<sup>59</sup>

**Effects of Immunotherapy**

Recent enhancements in our understanding of disease biology have led to the development of targeted therapies. With the introduction of CD19 directed therapies (chimeric antigen receptor [CAR-T] and BiTE [bispecific T cell engaging small molecules], blinatumomab), the MRD assessment by FCM has become more challenging. Studies have shown that these therapies can lead to downregulation of CD19 on

leukemic cells by different mechanisms, which minimize its utility to be used as gating marker.<sup>60,61</sup> In this regard, alternate markers have to be used to delineate the abnormal cells. A study by Cherian et al had shown that CD22 and CD24 may be used to detect MRD B-ALL after anti-CD19 therapy. The B cells are identified by the expression of CD22 or CD24 without CD66b and include cells that may or may not have CD19 positive B cells, but express either CD22 or CD24. Such populations may include some dendritic cell populations and basophils, and as well include CD34 positive progenitor populations. Other considerations are relevant while using these two markers for MRD analysis. With regard to hematogones, there will always be a maturation pattern for CD22 and CD24. However, the expression of CD22 is dim in the early hematogones and gradually increases till mature B cell stage. CD24, on the other hand, shows bright expression in hematogones and diminishes as the cell mature. Therefore, these markers have to be used in addition to CD10 and CD34 among others to avoid false negative or false positive results.<sup>62</sup> Inotuzumab ozogamicin is an anti-CD22 monoclonal antibody conjugated to calicheamicin, a cytotoxic agent and causes double-stranded DNA breaks initiating apoptosis.<sup>63</sup> Anti-CD22 therapy leads to decreased expression of CD22 on B cells. In these cases, gating using CD79a along with use of additional markers CD24/CD66b among others is done.<sup>64</sup>

FCM-based analysis in patients receiving CAR-T therapy needs to be done in CAR-T infusion bag and in PB samples. The assessment has to be done at various time points following the infusion with the knowledge that there is known variations in CAR-T cell percentage over time. Usually, a peak in CAR-T cell percentage occurs 12 days after the infusion. Although exact time point is still undetermined, analysis at day 1, day 7, day 14, and day 30 ( $\pm 2$  days for each time point) following the CAR-T infusion is done. In terms of CD4+ and CD8+ balance within CAR-T cell subset, CD4+ cells are the main lymphocytes shortly after the infusion, CD8+ lymphocyte subset increased later on to reach first an equilibrium with CD4+ subset and then become the most significant lymphocyte population within CAR-T cells.<sup>65</sup> Having said that, it is extremely important for a clinician to mention the targeted therapy used in a given patient while sending out the requisition forms to avoid false negative results.

## MRD in T-ALL

MRD is one of the key predictors of prognosis in T-ALL. MRD-based categorization of patients into low-risk allows them to be exposed to less intensive therapy and thus a lower risk of chemotherapy-related morbidity and mortality. MRD assessment is also useful in the setting of stem cell transplantation and for early recognition of impending relapse.<sup>66-68</sup>

### Time Points for MRD Detection

The time points of measurement of MRD detection in T-ALL also depend upon the protocol used in therapy: As with B-ALL, in modified BFM, it is done 4 weeks postinduction;

22 week at the end of reinduction and end of therapy. An alternate protocol prefers at 6 weeks postinduction and at the end of therapy. In India, many centers use the ICiCLE protocol in children which involves MRD assessment only postconsolidation (week 11).<sup>37,69,70</sup>

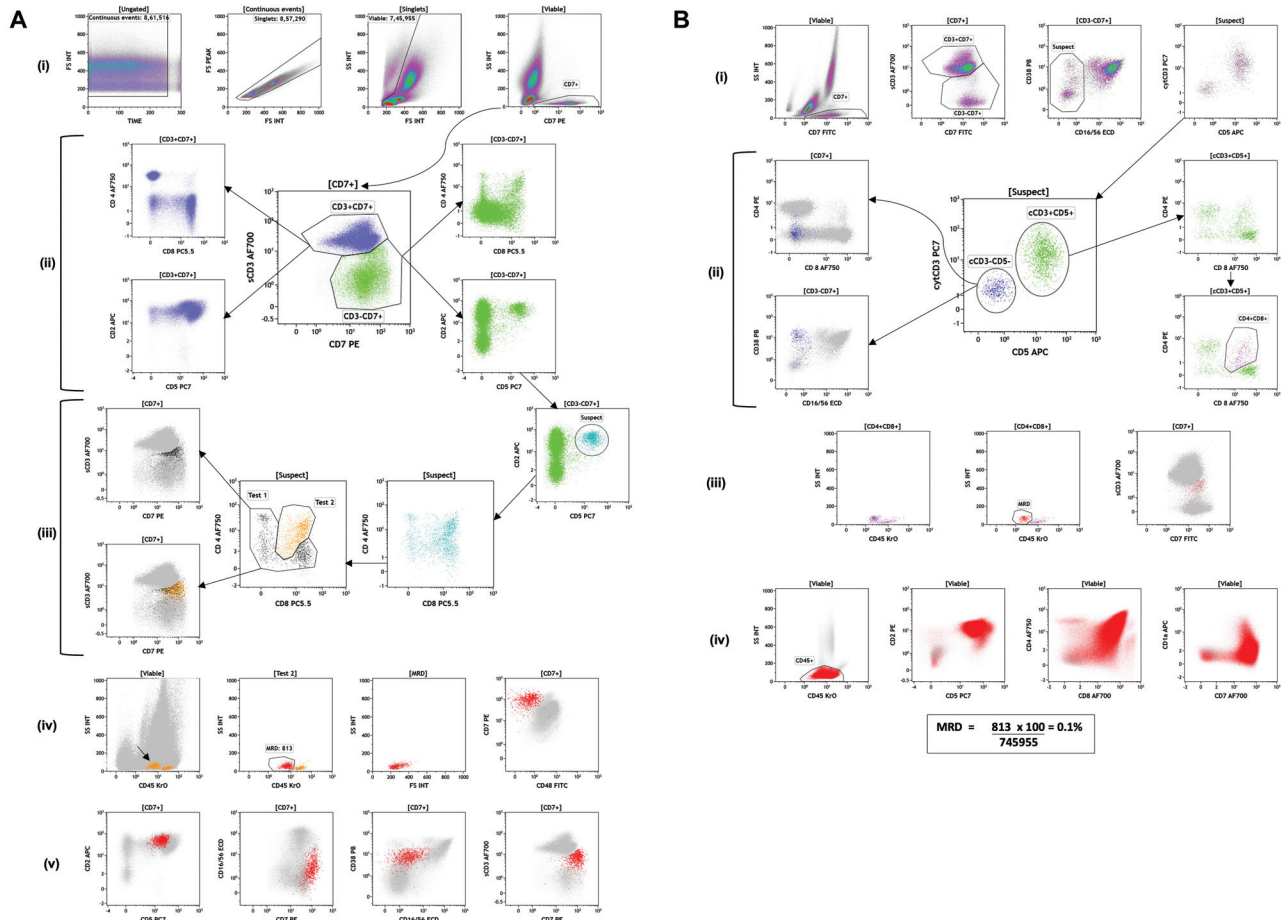
### Flow Cytometric MRD Analysis

Standard techniques for T-MRD include multiparametric FCM and PCR to detect clone-specific TCR gene and leukemia-specific fusion transcripts.<sup>14</sup> In contrast to precursor B-ALL, in T-ALL, PB sampling is equally useful as compared with BM sample and recent studies indicate that they have equal sensitivity in MRD detection. Precursor-B-ALL is of BM origin, which probably explains the higher MRD levels in BM than in the PB samples. T-ALL, on the other hand, is of thymic origin and shows a highly disseminating character, resulting in comparable MRD levels in BM and PB samples. PB blood MRD levels in T-ALL patients are comparable or up to 1 log lower than in BM.<sup>23,71</sup>

To find residual leukemic cells, it was initially suggested to look for immature T cells (cells co-expressing cCD3/TdT or CD7/TdT) outside of the thymus. However, it has been demonstrated that after treatment, the expression of immature antigens such TdT and CD99 is downregulated. A more effective strategy takes use of the difference in surface CD3 expression between mature and immature T cells, as well as leukemic T cells (**Fig. 3**). For T lineage ALL, the MRD assay should preferably comprise of CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD34, CD38, CD45, CD48, and CD56. Additional markers that may be used include CD1a, TdT, CD13, CD33, and CD117.<sup>72</sup> Mature T cells express a high level of surface CD3 and leukemic T cells often lack the surface expression but show a cytoplasmic CD3 expression. To achieve lineage specificity, cytoplasmic CD3 is combined with surface CD3, but to exclude the typically tiny subset of NK cells that may express cytoplasmic CD3, NK cell-associated antigens like CD56 and CD16 must also be included. Notably, immature NK cells are also CD16/56 negative. CD38, in combination of other T cell markers, helps in their exclusion. Aberrant T cells may show a lower antigen expression or complete absence of markers, such as CD5, sCD3, CD2, and dual negative or dual positivity of CD4 & CD8. Another frequent observation is the bright expression of CD7, and the expression of CD13/CD33 (lineage infidelity) can frequently aid in the identification of MRD.<sup>73-75</sup> Recently, a few new parameters have been studied for assessing the MRD in T-ALL. Median fluorescence intensity of CD48 was compared between normal T and NK cells and leukemic blasts that showed consistently decreased expression of CD48 on leukemic blasts which persisted even at postinduction phase of chemotherapy.<sup>76</sup>

### Measures of Analytical Sensitivity of MFC Assays

Establishing the sensitivity of an assay designed for rare event analysis, such as MRD detection, is of paramount importance. The introduction of concepts of limit of blank (LOB), limit of detection (LOD) and lower limit of



**Fig. 3** (A) MRD analysis from bone marrow aspirate of a case of T-ALL on therapy, using a 10-color and two-tube assay. These representative plots are from analysis of first tube comprising of CD48 FITC, CD7 PE, CD16/CD56 ECD, CD8 PC5.5, CD5 PC7, CD2 APC, CD3 APCA750 (Surface), CD4 APCA750, CD38 PB, and CD45 KrO. (i) The four biaxial density plots show gating of CD7 variably positive and side scatter (SS) low events (labeled as “CD7 +”), after sequentially gating the continuous, nondoublet (single cell) and viable events. (ii) Panel of plots where CD7+ events have been shown in the central biaxial dot plot of CD7 versus CD3 (surface), which reveals two major populations, one of which is surface-CD3 positive and CD7 variably positive, labeled as CD3 + CD7+ (blue dots), and the other population is surface-CD3 negative and CD7 positive, labeled as CD3-CD7+ (green dots). The CD3 + CD7+ population represents normal T lymphocytes and show normal profile of CD4, CD8, CD2, and CD5, as noted in CD4 versus CD8 and CD2 versus CD5 plots (left side plots). The CD3-CD7+ population predominantly revealed immunophenotypic profile consistent with NK cells, that is, CD4 and CD8 negative, CD5 negative and CD2 variably positive (right side plots). However, a small discrete subset showed dual CD2 and CD5 positivity. (iii) The CD2 and CD5 dual positive cells were gated (labeled Suspect). These cells showed heterogeneity in CD4 and CD8 expression and were further gated as test 1 (black dots) and test 2 (Orange dots). Test 1 population was distributed among CD4 and CD8 and was normal T cell with slight downregulation of surface CD3 expression. Test 2 population had a dual CD4 and CD8 positivity and showed a dim surface CD3 expression. (iv) Retrospective look in CD45 versus SS plot showed a subset of test 2 population with dim CD45 expression (arrow) which was gated as the residual leukemic cell population, labeled MRD (red dots). This population showed a tight cluster on FS versus SS plot. It also showed loss of CD48 expression with brighter CD7 expression than normal T cells on CD48 versus CD7 plot. (v) Retrospective analysis of residual leukemic cells with normal T/NK cells in the background. (B) Representative plots from analysis of second tube comprising of CD7 FITC, CD4 PE, CD16/CD56 ECD, CD34 PC5.5, CD3 PC7 (Cytoplasmic), CD5 APC, CD3 APCA750 (Surface), CD8 APCA750, CD38 PB, and CD45 KrO. (i) Biaxial density plots show gating of clean CD7+ events and further gating of two major subpopulations on CD7 versus surface CD3 plot. The CD3-CD7+ population is shown in CD16/CD56 versus CD38 plot with gating of CD16/CD56 negative and CD38 variable events (labeled as Suspect). This population has been shown in cytoplasmic-CD3 and CD5 plot that reveals two discrete subpopulations. (ii) Panel of plots where the “suspect” events have been shown in the central biaxial dot plot of CD5 versus cytoplasmic-CD3. The events labeled as cCD3-CD5- (blue dots) represent immature NK cells, noted further as CD4 and CD8 dual negative, and CD38 bright positive with absence of CD16/CD56. The cCD3 + CD5+ population (green dots) shows a cluster of CD4 and CD8 dual positive population in addition to normally distributed T cells. This dual-positive population is gated and labeled as CD4 + CD8+ (purple dots). (iii) The CD4 + CD8+ events reveal a CD45 dim cluster on CD45 versus SS plot which is further gated and labeled as MRD (red dots), noted to show a dimmer surface CD3 and a brighter CD7 expression than normal T cells, similar to that noted in first tube. (iv) Representative plots from the diagnostic sample showing CD45 dim blasts with dual positivity for CD2 and CD5 and for CD4 and CD8. The blasts also show a bright CD7 expression with only a minor population with CD1a expression. The text box shows percentage of MRD with numerator (MRD events) and denominator (viable events) calculated from the first tube.

quantification (LLOQ) in rare event analysis has been a remarkable advancement to ensure robust and reliable measurements of rare events. However, the LOB is the highest *apparent* analyte concentration which is expected to be found when multiple replicates of a blank sample containing no analyte are tested and are calculated as mean blank + 1.645 SD (standard deviation [blank]). LOD, on the other hand, is the lowest analyte concentration likely to be reliably distinguished from the LOB and is commonly defined as LOB + 1.645 SD (low positive samples). LOD is the level where 95% of low levels of events measure and are detected above LOB. LLOQ is the lowest level of measure and that can be reliably detected and the total error (bias and imprecision) meets a desired criterion for accuracy, defined on basis of clinical utility. The LLOQ is usually higher or at times equal to the LOD, but is never lower than it.<sup>77</sup> For rare event analysis like MRD assay, a strategy of replicate assay of negative samples as well as samples with low target population frequency can be used for estimating analytical sensitivity. For example, LOB can be established by acquiring five list mode files each from five replicates of five separate samples, which do not have target population of interest. LOB is confirmed when no more than 5% of the blank replicates exceed the low positive target. Similarly, LOD can be established by repeated assay of samples having low levels of the target population to determine SD and calculated imprecision. For clinical samples, 20 to 30 “relevant” events, with acquisition of one million or more clean events (devoid of debris and cell aggregates), are considered enough to classify rare population as “detectable,” and at least 50 events can make such population “quantifiable.” Increasing number of relevant events decreases CV, which is considered acceptable if around 10%, and increasing the total number of clean events increases the sensitivity of assay. The functional sensitivity or LLOQ may be determined by an approach in which serial dilutions of cells from a positive sample are spiked into a negative sample. It is then appropriate to verify a desired LLOQ by assaying at least five replicates near the LLOQ, each replicate analyzed and confirming that an acceptable level of imprecision is achieved.<sup>78,79</sup>

### Limitations of Flow Cytometry-Based MRD

One of the prime limitations of detection of residual cells by FCM is the immunophenotypic shifts that are observed as a result of chemotherapeutic reagents. While the targeted therapy mentioned above results in overall reduced expression of the antigens concerned, other conventional agents (alkylating drugs and steroids) cause subtle shifts in the antigenic profile in the normal BCPs, accurate interpretation of which poses a great amount of challenge.<sup>80</sup> Some of the notable shifts include brighter expression of CD10 on early hematogones and proliferation of type 1 hematogones in end of induction samples compared with postconsolidation and subsequent samples.<sup>81,82</sup> Leukemic cells also show some changes in the antigenic profile most importantly down-regulation of CD34 and CD10 and upregulation of CD19, CD20, and CD45.<sup>55,74</sup>

With respect to T-ALL, studies have reported decline in TdT and CD99, variable expression of CD10 and CD34 on leukemic blasts during therapy, while the lineage specific markers including CD2, CD3, CD4, CD5, CD7, and CD8 were relatively stable.<sup>74</sup>

Another important consideration includes the diluted sample that stresses upon the need to provide first pull of BM sample for achieving a high sensitivity MRD analysis.

The challenges mentioned above along with differences in antibody panel design and fluorochrome combination complicate interlaboratory standardization. Improved participation in external quality programs, like those provided by United Kingdom National External Quality Assessment Scheme (UKNEQAS) and by the College of American Pathologists (CAP), can help to improve and sustain quality of the assay. In India, an interlaboratory comparison program (ILCP) is being run by the Department of Hematopathology, ACTREC, Tata Memorial Centre, Navi Mumbai.

### Future Considerations

Past decade has witnessed a plethora of advancements in methodologies for the detection of MRD that aim at higher sensitivity and broader applicability. EuroFlow Consortium has developed a high throughput “next-generation flow cytometry” which analyzes the data on multivariate plots wherein a test sample is compared with that of the standard one. A revolutionary piece of software, like Infinicyt, gauges how far cells in the test sample have deviated from the standard in terms of cell maturation and differentiation. Regular QA evaluations have completely standardized this method.<sup>83,84</sup>

Another breakthrough in MRD quantification is the NGS. EuroClonality-NGS Consortium has made guidelines/workflow as well as standardization for NGS-based Ig/TCR assays in hemato-oncology. This method entails identifying clonal Ig/TCR gene rearrangements that are unique to leukemia in the diagnostic sample. The identical set of primers as for RQ-PCR is used. These sequences are then employed for the detection of MRD in the subsequent follow-up samples. Compared with Sanger sequencing-based assays, NGS has a quicker turnaround time and greater sensitivity (down to even  $10^{-7}$ ), although this depends heavily on the capability of the laboratory. However, the amount of input DNA necessary to attain this high level of sensitivity should be substantial (~65 µg), which is difficult in postinduction marrow samples. Although becoming increasingly popular for acute myeloid leukemia, this technique is in its nascent stage to be implicated as a routine investigation in ALL patients.<sup>85-87</sup>

### Conclusions

To conclude, detection of MRD using FCM has been in practice since 1990s and has superseded the light microscopy with much enhanced sensitivity. As the protocols have been standardized, there have been significant advancements in risk assessment and prognosis resulting in even more improvement in childhood and adult ALL cure rates.



Lymphocyte profiling and myriad of immunophenotypic markers allow quick and reliable assessment of MRD in ALL. In the light of recent development of novel and targeted therapies in ALL, the FCM-based MRD analysis has garnered even broader applicability. The EuroFlow consortium has developed new high-throughput concepts for flow MRD of ALL employing an eight-color single tube panel based on multivariate analysis, with the use of newer fluorochrome combinations projected to rise in the future decade. This would help to increase the applicability and specificity of measurements of flow MRD.

#### Conflict of Interest

None declared.

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